PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 13 June 2001 (13.06.01)	
International application No. PCT/GB00/03360	**Applicant's or agent's file reference** N.77069A GCW
International filing date (day/month/year) 31 August 2000 (31.08.00)	**Priority date** (day/month/year) 31 August 1999 (31.08.99)
Applicant GARTHWAITE, Giti et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

19 March 2001 (19.03.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia TEFY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

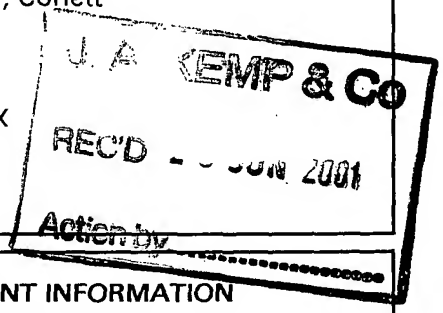
PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

To:

WOODS, Geoffrey, Corlett
J.A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
ROYAUME-UNI



INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

Date of mailing (day/month/year) 13 June 2001 (13.06.01)	
Applicant's or agent's file reference N.77069A GCW	**IMPORTANT INFORMATION**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/GB00/03360	31 August 2000 (31.08.00)	31 August 1999 (31.08.99)

Applicant
UNIVERSITY COLLEGE LONDON et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,CA,CN,CZ,DE,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   AP :GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZW
   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
   National :AE,AG,AL,AM,AT,AZ,BA,BB,BR,BY,BZ,CH,CR,CU,DK,DM,DZ,EE,ES,FI,GB,
   GD,GE,GH,GM,HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MW,
   MX,MZ,PT,SD,SG,SI,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:
Olivia TEFY

Telephone No. (41-22) 338.83.38

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ EPO

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: N.77069A GCW

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/GB00/03360	31 August 2000 (31.08.00)	31 August 1999 (31.08.99)

Title of invention

SCREEN FOR AXON VIABILITY

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

UNIVERSITY COLLEGE LONDON
Gower Street
London
WC1E 6BT
United Kingdom

Telephone No.:

Facsimile No.:

Teleprinter No.:

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
GB	GB

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

GARTHWAITE Giti
The Wolfson Institute for Biomedical Research
The Cruciform Building
University College London
Gower Street
London WC1E 6BT
United Kingdom

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
GB	GB

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

GARTHWAITE John
The Wolfson Institute for Biomedical Research
The Cruciform Building
University College London
Gower Street
London WC1E 6BT
United Kingdom

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
GB	GB

☐ Further applicants are indicated on a continuation sheet.

International application No.
PCT/GB00/03360

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is [x] agent [ ] common representative

and [x] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

WOODS Geoffrey Corlett
J.A. KEMP & CO.
14 South Square
Gray's Inn
London
WC1R 5JJ
United Kingdom

Telephone No.: +44 20 7405 3292

Facsimile No.: +44 20 7242 8932

Teleprinter No.: 23676

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special addr ess to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination **to start on the basis of:**

[x] the international application as originally filed

the description [ ] as originally filed
[ ] as amended under Article 34

the claims [ ] as originally filed
[ ] as amended under Article 19 (together with any accompanying statement)
[ ] as amended under Article 34

the drawings [ ] as originally filed
[ ] as amended under Article 34

2. [ ] The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. [ ] The applicant wishes the start of the international preliminary examination **to be postponed** until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

[x] which is the language in which the international application was filed.

[ ] which is the language of a translation furnished for the purposes of international search.

[ ] which is the language of publication of the international application.

[ ] which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby **elects all eligible States** *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes **not to elect:**

International application No.
PCT/GB00/03360

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	not received
1. translation of international application	:	sheets	☐	☐
2. amendments under Article 34	:	sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	:	sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. [x] fee calculation sheet
2. [ ] separate signed power of attorney
3. [ ] copy of general power of attorney; reference number, if any:
4. [ ] statement explaining lack of signature
5. [ ] nucleotide and or amino acid sequence listing in computer readable form
6. [ ] other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

WOODS, Geoffrey Corlett

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

See Notes to the demand form

CHAPTER II

PCT

FEE CALCULATION SHEET

Annex to the Demand for international preliminary examination

International application No.	PCT/GB00/03360
Applicant's or agent's file reference	N.77069A GCW

For International Preliminary Examining Authority use only

Date stamp of the IPEA

Applicant

UNIVERSITY COLLEGE LONDON

Calculation of prescribed fees

1. Preliminary examination fee	EUR 1533	P
2. Handling fee *(Applicants from certain States are entitled to a reduction of 75% of the handling fee. Where the applicant is (or all applicants are) so entitled, the amount to be entered at H is 25% of the handling fee.)*	EUR 147	H
3. Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box.....	EUR 1680	TOTAL

Mode of Payment

[X]	authorization to charge deposit account with the IPEA (see below)	[ ]	cash
[ ]	cheque	[ ]	revenue stamps
[ ]	postal money order	[ ]	coupons
[ ]	bank draft	[ ]	other *(specify)*:

Deposit Account Authorization *(this mode of payment may not be available at all IPEAs)*

The IPEA/ EPO [ ] is hereby authorized to charge the total fees indicated above to my deposit account.

[ ] *(this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

2805.0038	16 March 2001	
Deposit Account Number	Date *(day/month/year)*	Signature WOODS, Geoffrey Corlett

See Notes to the fee calculation sheet

TENT COOPERATION TREA

PCT

From the INTERNATIONAL BUREAU

To:

WOODS, Geoffrey, Corlett
J.A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
ROYAUME-UNI

J. A. KEMP & Co
REC'D 10 NOV 2000
Action by.....

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 02 November 2000 (02.11.00)	
Applicant's or agent's file reference N.77069A GCW	IMPORTANT NOTIFICATION
International application No. PCT/GB00/03360	International filing date (day/month/year) 31 August 2000 (31.08.00)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 31 August 1999 (31.08.99)
Applicant UNIVERSITY COLLEGE LONDON et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. *This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.*

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
31 Augu 1999 (31.08.99)	9920566.8	GB	27 Sept 2000 (27.09.00)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Maria Victoria CORTIELLO

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
WOODS, Geoffrey, Corlett
J.A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
ROYAUME-UNI

Date of mailing (day/month/year) 08 March 2001 (08.03.01)	
Applicant's or agent's file reference N.77069A GCW	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/GB00/03360	31 August 2000 (31.08.00)	31 August 1999 (31.08.99)

Applicant
UNIVERSITY COLLEGE LONDON et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AG,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CR,CU,CZ,DE,DK,DM,DZ,EA,EE,EP,ES,
   FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,
   MN,MW,MX,MZ,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   08 March 2001 (08.03.01) under No. WO 01/16359

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum) N.77069A GCW

Box No. I TITLE OF INVENTION

SCREEN FOR AXON VIABILITY

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

UNIVERSITY COLLEGE LONDON
Gower Street
London
WC1E 6BT
United Kingdom

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

GARTHWAITE Giti
The Wolfson Institute for Biomedical Research
The Cruciform Building
University College London
Gower Street
London WC1E 6BT
United Kingdom

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

WOODS Geoffrey Corlett
J.A. KEMP & CO.,
14 South Square,
Gray's Inn,
London, WC1R 5LX,
United Kingdom.

Telephone No.
+44 20 7405 3292

Facsimile No.
+44 20 7242 8932

Teleprinter No.
23676

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

GARTHWAITE John
The Wolfson Institute for Biomedical Research
The Cruciform Building
University College London
Gower Street
London WC1E 6BT
United Kingdom

This person is:

☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

☒ **AP** **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, MZ Mozambique, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☒ **EA** **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

☒ **EP** **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ **OA** **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

☒ **AE** United Arab Emirates
☒ **AG** Antigua and Barbuda
☒ **AL** Albania .....
☒ **AM** Armenia .....
☒ **AT** Austria .....
☒ **AU** Australia .....
☒ **AZ** Azerbaijan
☒ **BA** Bosnia and Herzegovina .....
☒ **BB** Barbados
☒ **BG** Bulgaria .....
☒ **BR** Brazil .....
☒ **BY** Belarus .....
☒ **BZ** Belize
☒ **CA** Canada
☒ **CH and LI** Switzerland and Liechtenstein
☒ **CN** China .....
☒ **CR** Costa Rica .....
☒ **CU** Cuba .....
☒ **CZ** Czech Republic .....
☒ **DE** Germany .....
☒ **DK** Denmark .....
☒ **DM** Dominica
☒ **DZ** Algeria .....
☒ **EE** Estonia .....
☒ **ES** Spain .....
☒ **FI** Finland .....
☒ **GB** United Kingdom
☒ **GD** Grenada
☒ **GE** Georgia .....
☒ **GH** Ghana .....
☒ **GM** Gambia
☒ **HR** Croatia .....
☒ **HU** Hungary .....
☒ **ID** Indonesia
☒ **IL** Israel .....
☒ **IN** India .....
☒ **IS** Iceland
☒ **JP** Japan .....
☒ **KE** Kenya .....
☒ **KG** Kyrgyzstan .....
☒ **KP** Democratic People's Republic of Korea .....
☒ **KR** Republic of Korea .....
☒ **KZ** Kazakhstan .....
☒ **LC** Saint Lucia
☒ **LK** Sri Lanka
☒ **LR** Liberia
☒ **LS** Lesotho .....
☒ **LT** Lithuania
☒ **LU** Luxembourg
☒ **LV** Latvia
☒ **MA** Morocco .....
☒ **MD** Republic of Moldova .....
☒ **MG** Madagascar .....
☒ **MK** The former Yugoslav Republic of Macedonia .....
☒ **MN** Mongolia
☒ **MW** Malawi .....
☒ **MX** Mexico .....
☒ **MZ** Mozambique
☒ **NO** Norway
☒ **NZ** New Zealand .....
☒ **PL** Poland .....
☒ **PT** Portugal .....
☒ **RO** Romania
☒ **RU** Russian Federation .....
☒ **SD** Sudan
☒ **SE** Sweden
☒ **SG** Singapore
☒ **SI** Slovenia .....
☒ **SK** Slovakia .....
☒ **SL** Sierra Leone .....
☒ **TJ** Tajikistan .....
☒ **TM** Turkmenistan .....
☒ **TR** Turkey .....
☒ **TT** Trinidad and Tobago .....
☒ **TZ** United Republic of Tanzania
☒ **UA** Ukraine .....
☒ **UG** Uganda .....
☒ **US** United States of America .....
☒ **UZ** Uzbekistan .....
☒ **VN** Viet Nam .....
☒ **YU** Yugoslavia .....
☒ **ZA** South Africa .....
☒ **ZW** Zimbabwe .....

Check-box reserved for designating States which have become party to the PCT after issuance of this sheet:

☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country	regional application:* regional Office	international application: receiving Office
item (1) 31 August 1999	9920566.8	United Kingdom		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): (1)

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)* :

ISA /

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	: 4
description (excluding sequence listing part)	: 22
claims	: 2
abstract	: 1
drawings	: 3
sequence listing part of description	:
Total number of sheets	: 32

This international application is **accompanied** by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☒ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other *(specify)*: PF 23/77

Figure of the drawings which should accompany the abstract: -

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

WOODS, Geoffrey Corlett

For receiving Office use only

1. Date of actual receipt of the purported international application:	2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority (if two or more are competent): ISA /	6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET
Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference N.77069A GCW

Applicant
UNIVERSITY COLLEGE LONDON

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . £55 T

2. SEARCH FEE . . . . . £605 S

International search to be carried out by _____
(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee
The international application contains 32 sheets.

first 30 sheets £264 b1

_____ x £6 = £12 b2
remaining sheets additional amount

Add amounts entered at b1 and b2 and enter total at B . . . . £276 B

Designation Fees
The international application contains >8 designations.

8 x £56 = £448 D
number of designation fees payable *(maximum 8)* amount of designation fee

Add amounts entered at B and D and enter total at I . . . . . £724 I

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* . . . . . £22 P

5. TOTAL FEES PAYABLE . . . . . £1406 TOTAL
Add amounts entered at T, S, I and P, and enter total in the TOTAL box

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

☐ authorization to charge deposit account (see below)	☐ bank draft	☐ coupons
☒ cheque	☐ cash	☐ other *(specify):*
☐ postal money order	☐ revenue stamps	

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ _____ ☐ is hereby authorized to charge the total fees indicated above to my deposit account.

☐ *(this check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

Deposit Account No.	Date *(day/month/year)*	Signature
	31 August 2000	WOODS Geoffrey Corlett

See Notes to the fee calculation sheet

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:
J.A. KEMP & CO.
Attn. WOODS, G.
14 South Square
Gray's Inn
London WC1R 5LX
UNITED KINGDOM

J. A. KEMP & Co
REC'D 25 JUN 2001
Action by.....

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of mailing *(day/month/year)* 22/06/2001
Applicant's or agent's file reference N.77069A GCW	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/GB 00/03360	International filing date *(day/month/year)* 31/08/2000
Applicant UNIVERSITY COLLEGE LONDON et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Geertruida Groeneveld-Van der Spek

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
N.77069A GCW		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/ 03360	31/08/2000	31/08/1999
Applicant		
UNIVERSITY COLLEGE LONDON et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/03360

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/527 G01N33/53 A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XIE XINMIN ET AL: "Interaction of the antiepileptic drug lamotrigine with recombinant rat brain type IIA Na+ channels and with native Na+ channels in rat hippocampal neurones." PFLUEGERS ARCHIV EUROPEAN JOURNAL OF PHYSIOLOGY, vol. 430, no. 3, 1995, pages 437-446, XP000993077 ISSN: 0031-6768 cited in the application abstract --- -/--	12-19

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 April 2001	22/06/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Pellegrini, P

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	XIE X M ET AL: "State-dependent inhibition of Na+ currents by the neuroprotective agent 619C89 in rat hippocampal neurons and in a mammalian cell line expressing rat brain type IIA Na+ channels." NEUROSCIENCE, vol. 73, no. 4, 1996, pages 951-962, XP000993069 ISSN: 0306-4522 cited in the application abstract	12-19
X	MELDRUM B S ET AL: "Reduction of glutamate release and protection against ischemic brain damage by BW 1003C87." BRAIN RESEARCH, vol. 593, no. 1, 1992, pages 1-6, XP000993068 ISSN: 0006-8993 cited in the application abstract	12-19
P,X	GARTHWAITE GITI ET AL: "Nitric oxide stimulates cGMP formation in rat optic nerve axons, providing a specific marker of axon viability." EUROPEAN JOURNAL OF NEUROSCIENCE, vol. 11, no. 12, December 1999 (1999-12), pages 4367-4372, XP000990808 ISSN: 0953-816X the whole document	1-19
P,X	GARTHWAITE G ET AL: "Monitoring rat optic nerve axon viability using nitric oxide-stimulated cGMP accumulation: Application to the mechanism of ischaemic damage." SOCIETY FOR NEUROSCIENCE ABSTRACTS., vol. 25, no. 1-2, 1999, page 1841 XP000990791 29th Annual Meeting of the Society for Neuroscience.;Miami Beach, Florida, USA; October 23-28, 1999 ISSN: 0190-5295 the whole document	1-19

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CECIL KIM M ET AL: "Proton magnetic resonance spectroscopy for detection of axonal injury in the splenium of the corpus callosum of brain-injured patients." JOURNAL OF NEUROSURGERY, vol. 88, no. 5, May 1998 (1998-05), pages 795-801, XP000990904 ISSN: 0022-3085 abstract	1-19
A	SANGER J R ET AL: "HISTOCHEMICAL STAINING OF NERVE ENDINGS AS AN AID TO FREE MUSCLE TRANSPLANTATION" MICROSURGERY, vol. 12, no. 5, 1991, pages 361-366, XP000990903 ISSN: 0738-1085 abstract	1-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 00/03360

B x I Observations wher certain claims were found unsearchable (Continuation of it m 1 of first she t)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 12-19
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 12-19

Claims 12-19 relate to substances defined by reference to a desirable characteristic or property, namely being identified by the method of claim 11, i.e. contacting an axon with a test substance under conditions that in the absence of the test substance would lead to a decrease in viability, determining the viability of the axon by a method according to claims1-10, and determining thereby whether the test substance can protect the axon from loss of viability. Claims 12-19 relate furthermore to medical uses and methods of treatment related to these substances. Claims 12-19 cover all substances having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for no such substances.
Furthermore, even known substances such as lamotrigine (Xie et al. (1995), Pfleugers Arch. Eur. J. Physiol. 430, 437-446), compound 619C89 (Xie et al. (1996), Neuroscience 73, 951-962) and BW 1003C87 (Meldrum et al. (1992), Brain Research 593, 1-6) fall under the scope of the claims. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the subject-matter for which protection is sought in claims 12-19 is impossible.
Consequently, no complete search has been performed on these claims.

It is also pointed out that claims 18-19 relate to treatment of human or animal body by therapy (Rule 39.1(iv) PCT).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

PATENT COOPERATION TREATY

MCC

PCT

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

WOODS, Geoffrey, Corlett
J.A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
GRANDE BRETAGNE

J. A. KEMP & Co
- 9 APR 2001
Action by

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence and Administrative Instructions, Section 601(a))

Applicant's or agent's file reference N.77069A GCW	Date of mailing *(day/month/year)* 05.04.01
	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB 00/03360	31/08/2000	31/08/1999

Applicant

UNIVERSITY COLLEGE LONDON et al.

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

19/03/2001

2. This date of receipt is:

- [X] the actual date of receipt of the demand by this Authority (Rule 61.1(b)).
- [ ] the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).
- [ ] the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. [ ] **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

[ ] *(If applicable)* This notification confirms the information given by telephone, facsimile transmission or in person on: _____

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	MORENO R A Tel. (+49-89) 2399-2658

EUROPÄISCHES PATENTAMT · EUROPEAN PATENT OFFICE · OFFICE EUROPÉEN DES BREVETS

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

WOODS, Geoffrey, Corlett
J.A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5LX
GRANDE BRETAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	09.11.2001
Applicant's or agent's file reference N.77069A GCW	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/03360	31/08/2000	31/08/1999

Applicant
UNIVERSITY COLLEGE LONDON et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Digiusto, M Tel.+49 89 2399-8162

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
N.77069A GCW		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/03360	31/08/2000	31/08/1999
International Patent Classification (IPC) or national classification and IPC C12Q1/527		
Applicant UNIVERSITY COLLEGE LONDON et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
19/03/2001	09.11.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Knudsen, H Telephone No. +49 89 2399 8696

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/03360

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-22	as originally filed			

Claims, No.:

1-20	as received on	25/10/2001	with letter of	25/10/2001

Drawings, sheets:

1/3-3/3	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 13-20.

because:

   ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion

could be formed.

☒ no international search report has been established for the said claims Nos. 13-20.

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-12
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-12
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-12
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
s e separate sheet

Re Item II
Priority

The priority appears to be allowable for all of the claimed subject-matter and the P-documents mentioned in the International Search Report therefore do not appear to be relevant.

Re Item III
Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

As explained in the International Search Report, the subject-matter of claims 13-20 was not searched and therefore is not examined.

Re Item V
Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following document:

D1: MELDRUM B S ET AL: 'Reduction of glutamate release and protection against ischemic brain damage by BW 1003C87.' BRAIN RESEARCH, vol. 593, no. 1, 1992, pages 1-6, ISSN: 0006-8993

NOVELTY & INVENTIVE STEP:

5.1 D1 discloses a method of measuring whether a drug is capable of inhibiting brain damage, the effect of the drug is determined via glutamate release measurement. The in-vitro method involves the steps of cutting a slice of brain and incubating the slice with veratrine hydrochloride or buffer and a test drug; glutamate is measured in the supernatant. In the in-vivo method, living rats are given drug and veratrine and glutamate is measured in a dialysis fluid. Glutamate release is expected to be an indicator of brain damage upon ischemic events (see page 5, left column).

The method of claim 1 differs from D1 in that the activity of sGC is measured. As explained by the applicant, the assay of present claim 1 does not determine sGC activity as a measure of endogenously produced NO or glutamate (ie sGC activation as a result of glutamate production). The present assay determines sGC activity in the axon upon activation with NO or glutamate. This concept is different from that used in D1. In addition, the method of D1 is carried out on brain tissue whereas the method of claim 1 is carried out on axons (ie white matter). Finally the applicant mentions that a link between glutamate concentration and sGC activation has not been shown in white matter and would not be expected because axons lack glutamate receptors and because immunocytochemical studies for sGC and cGMP showed poor staining of white matter.

The IPEA agrees on the basis of this explanation that the skilled person would not be guided to the assay of present claim 1 by D1. Thus, claim 1 and its dependencies (ie claims 2-12) are considered novel and inventive.

INDUSTRIAL APPLICABILITY:

5.2 Present claims 1-12 are directed to an in-vitro method carried out on axons outside the body and claims 1-12 are therefore considered industrially applicable.

Re Item VII

Certain defects in the international application

7.1 Contrary to the requirements of Rule 5(a)(ii) PCT, the relevant background art disclosed in D1 is not briefly discussed in the description.

7.2 Contrary to the PCT Guidelines C-II 4.16-4.17, registered trade marks have not been identified as such in the description.

Re Item VIII

Certain observations on the international application

8.1 The applicant refers to white matter as the substance being tested with the claimed method. However, the use of white matter axon is not a feature of independent claim 1.

8.2 The applicant argues that the claimed method provides advantages over the histological techniques used in the prior art. Nevertheless, the claimed method seems to encompass the use of histology for detecting sGC or cGMP.

absence of the test substance would lead to a decrease in viability;

(ii) determining the viability of the axon by a method according to any one of the preceding claims; and

(iii) determining thereby whether the test substance can protect the axon from loss of viability.

13. A substance identified by a method according to claim 12.

14. A substance according to claim 13 for use in a method of treatment of the human or animal body by therapy.

15. A substance according to claim 14 for use in a method of treatment of a condition associated with white matter damage.

16. A substance according to claim 15 for use in a method of treatment of cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease.

17. Use of a substance according to claim 12 in the manufacture of a medicament for use in the treatment of a condition associated with white matter damage.

18. Use of a substance according to claim 12 in the manufacture of a medicament for use in the treatment of cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease.

19. A method of treating a host suffering from a condition associated with white matter damage, which method comprises administering to the host a therapeutically effective amount of a substance according to claim 12.

20. A method of treating a host suffering from cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease, which method comprises administering to the host a therapeutically effective amount of a substance according to claim 12.

CLAIMS

1. A method for determining the viability of an axon comprising:

(i) contacting the axon *ex vivo* with a substance that is capable of stimulating soluble guanylate cyclase (sGC);

(ii) determining whether sGC is stimulated in the axon; and

(iii) determining thereby whether the axon is viable.

2. A method according to claim 1, wherein step (i) is carried out in a physiologically acceptable buffer.

3. A method according to claim 1 or 2, wherein the axon is a white matter axon.

4. A method according to claim 3, wherein the white matter axon is from the optic nerve, the brain or the spinal cord.

5. A method according to any one of the preceding claims, wherein step (i) is carried out by contacting the axon with nitric oxide (NO), 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), cardon monoxide (CO) or YC-1 and CO.

6. A method according to claim 5 wherein NO is provided in the form of an NO donor.

7. A method according to claim 6, wherein the NO donor is 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO).

8. A method according to any one of the previous claims, wherein step (ii) is carried out by determining whether cGMP generation by the axon increases.

9. A method according to claim 8, wherein the generation of cGMP is determined by radioimmunoassay or immunocytochemistry.

10. A method according to claim 8 or 9, wherein a viable axon is one which shows a greater increase in cGMP generation than that shown by a non-viable axon.

11. A method according to claim 10, wherein the increase in cGMP production is at least 2-fold that shown by a non-viable axon.

12. A method for identifying a substance capable of protecting an axon from loss of viability comprising:

(i) contacting an axon with a test substance under conditions that in the

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: SCREEN FOR AXON VIABILITY

(57) Abstract: A method for determining the viability of an axon comprises: (i) contacting the axon with a substance that is capable of stimulating soluble guanylate cyclase (sGC); (ii) determining whether sGC is stimulated in the axon; and (iii) determining thereby whether the axon is viable.

SCREEN FOR AXON VIABILITY

Technical field of the invention

This invention relates to methods for assaying for axon viability and to methods for screening for substances which protect axons from loss of viability.

Background to the invention

Axons in CNS white matter become damaged in various debilitating conditions affecting humans, including stroke, trauma and multiple sclerosis (Stys, 1998; Trapp et al., 1998). The underlying mechanisms, however, have not been investigated as extensively as those causing damage to grey matter. In part at least, this is attributable to the technical difficulties of studying white matter pathology. The available information on white matter axons has so far come mainly from electrophysiological experiments on the rat isolated optic nerve preparations, in which the degree of recovery of the compound action potential following transient anoxia is used as an index of viability (Stys, 1998). A similar method has been applied to traumatic damage in the spinal cord (Agrawal & Fehlings, 1996).

A quantitative morphometric approach for analysing white matter axon pathology has recently been developed and used to study the mechanisms of rat optic nerve axon degeneration resulting from transient oxygen- and glucose-deprivation (OGD) *in vitro* (Garthwaite *et al.*, 1999). The results suggest a mechanism similar to that proposed to explain anoxic axonal damage (Stys, 1998), namely that excessive influx of Na^+ through voltage-dependent Na^+ channels is followed by lethal Ca^{2+} overload of the axoplasm through reversal of the Na^+-Ca^{2+}-exchanger located in the cell membrane. The histological method, however, suffers from the disadvantage of not recording axonal function and so interpretations based purely on morphological criteria may be misleading.

Nitric oxide (NO) functions as a diffusible second messenger molecule in most areas of the central nervous system (CNS). It is generated from L-arginine by NO synthase enzymes, the neuronal isoform of which is functionally and physically associated with the N-methyl-D-aspartate type of glutamate receptor in many brain

areas (Garthwaite & Boulton, 1995; Christopherson & Bredt, 1997). A major mechanism for NO signal transduction is activation of the enzyme soluble guanylyl cyclase (sGC), which causes the formation of cGMP from guanosine 5'-triphosphate (GTP). This pathway appears to mediate many of the physiological actions of NO in the CNS and elsewhere (Ignarro, 1991; Garthwaite & Boulton, 1995; Christopherson & Bredt, 1997; Hobbs, 1997).

Summary of the invention

We have unexpectedly found that the rat optic nerve, a CNS white matter tract which lacks synapses and is composed mainly of glial cells and axons, is capable of generating large quantities of cGMP in response to NO and that this response is confined to the axons. This discrete localization, together with the fact that cGMP formation requires high energy phosphates that are lacking in non-viable tissue, indicated that the response can serve as a sensitive marker for optic nerve axon viability.

The finding that NO leads to cGMP formation in optic nerve cell axons is surprising. Previous evidence has indicated that, in the CNS, the NO-cGMP signalling pathway is primarily associated with synapses, yet synapses are absent from the optic nerve. Also, the neurones giving rise to the optic nerve axons, the retinal ganglion cells, do not appear to react to NO in the same way. In bovine or rat retinae, little or no cGMP immunostaining was observed in these cells in response to NO-donor compounds.

According to the present invention there is thus provided a method for determining the viability of an axon comprising:

(i) contacting the axon with a substance that is capable of stimulating soluble guanylate cyclase (sGC);

(ii) determining whether sGC is stimulated in the axon; and

(iii) determining thereby whether the axon is viable.

The invention also provides:

– a method for identifying a substance capable of protecting an axon from loss

of viability comprising:

(i) contacting an axon with a test substance under conditions that in the absence of the test substance would lead to a decrease in viability;

(ii) determining the viability of the axon by a method according to any one of the preceding claims; and

(iii) determining thereby whether the test substance can protect the axon from loss of viability;

- a substance identified by a method for identifying a substance capable of protecting an axon from loss of viability;
- a substance of the invention for use in a method of treatment of the human or animal body by therapy;
- use of a substance of the invention in the manufacture of a medicament for use in the treatment of a condition associated with white matter damage;
- use of a substance of the invention in the manufacture of a medicament for use in the treatment of cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease;
- a method of treating a host suffering from a condition associated with white matter damage, which method comprises administering to the host a therapeutically effective amount of a substance of the invention; and
- a method of treating a host suffering from cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease, which method comprises administering to the host a therapeutically effective amount of a substance of the invention.

Brief description of the figures

Figure 1 shows (a) DEA/NO concentration-response curve for cGMP

accumulation in isolated adult rat optic nerves. (b) Protection of the cGMP response to 100 μM DEA/NO of OGD-treated optic nerves (shaded columns) by removal of Ca^{2+} ($0Ca^{2+}$) or Na^{+} ($0Na^{+}$) or addition of TTX (1 μM). All 3 treatments significantly restored cGMP level (P <0.001). Data are means ± S.E.M (n = 4-9).

Figure 2 shows protection against OGD-induced loss of optic nerve cGMP response to 100 μM DEA/NO by lamotrigine and analogues. Nerves kept in aCSF throughout are indicated by the open columns; nerves subjected to OGD are shown in shaded columns; *P < 0.02; **P < 0.0001 versus OGD alone (n = 4-12).

Figure 3 shows the histology and cGMP immunohistochemistry in control and OGD-treated optic nerves. (a) Semithin longitudinal section of untreated optic nerve following 5 h incubation. (b,c) cGMP immunostaining in longitudinal frozen sections of nerves incubated without (b) or with (c) DEA/NO for 5 min. (d-f) Semithin sections showing control histology in a transversely-cut optic nerve (d) and cGMP immunostaining in transverse (e) and longitudinal (f) sections of DEA/NO-treated nerves. (g-i) Semithin cross-sections showing the histology of optic nerves subjected to 1 h of OGD in the absence (g) and presence of BW619C89 (100 μM, h), or 1 μM TTX (i) followed, in each case, by 90 min recovery in normal aCSF. (j-l) cGMP immunohistochemistry of longitudinal frozen sections from DEA/NO-stimulated nerves previously subjected to 1 h OGD in the absence (j) or presence of BW619C89 (100μM, k) or TTX (1 μM, l). The DEA/NO concentration was 100 μM in all cases. Key: short arrows, axons; large arrowhead, oligodendrocyte; double small arrowheads, astrocyte soma; open arrows, band of glial cells; curved arrow, astrocyte processes. Scale bar (10 μm shown in a) applies to all micrographs.

Detailed description of the invention

The present invention provides a method for determining the viability of an axon which consists essentially of the following steps:

(i) contacting the axon with a substance that is capable of stimulating soluble guanylate cyclase (sGC);

(ii) determining whether sGC is stimulated in the axon; and

(iii) determining thereby whether the axon is viable.

This assay for axon viability is significant, as no other simple methods for assessing white matter axon viability are presently available.

In principle the assay for determining the viability of an axon may be carried out to determine the viability of any axon. However, the assay is particularly suitable for determining the viability of white matter axons. White matter is an area of the nervous system, containing abundant myelinated axons and is therefore light in colour. The central nervous system comprising the brain and spinal cord and the peripheral nervous system both contain white matter and axons from these sources may be used in the assay of the invention. Axons from the optic nerve are particularly suitable.

In principle the assay may be carried out using a single axon. However, in practice it is more convenient to use more than one axon in a single assay. Typically, a population of axons, for example a nerve, is used. The viability determined when more than one axon is used will represent an average viability for the population of axons used.

In viable axons, NO activates sGC, leading to an increase in cGMP formation, which in turn leads to the modulation of the activity of a number of cGMP targets. A viable axon may thus be identified by determining whether this pathway is functional in that axon. The activity of sGC before and after contacting an axon with a substance capable of stimulating sGC may be determined in order to determine whether sGC activity is stimulated, thereby to determine whether the axon is viable.

Any suitable format may be used for carrying out the assay of the invention. Generally, the assay is carried out *ex vivo* and under physiologically acceptable conditions; that is, under conditions that would be expected to support axon survival. It will often be convenient to carry out the assay in an aqueous medium, for example a physiologically acceptable buffer.

Typically, the assay is initiated by contacting an axon with a substance that is capable of stimulating sGC. Such a substance is generally one which under normal physiological conditions is capable of activating sGC in a viable axon. Suitable

activators of sGC include nitric oxide (NO), 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), carbon monoxide (CO) or YC-1 and CO. A combination of YC-1 and CO is a very effective activator of sGC.

Stimulators of sGC may be supplied in any way. For example, NO may be supplied in the form of an NO donor. This is particularly suitable if the assay is carried out in an aqueous environment. Suitable NO donors include organic nitrates (eg. glyceryl trinitrate), nitrites (eg. amyl nitrite), inorganic nitroso compounds (eg. sodium nitroprusside), sydnonimines (eg. molsidomine, 3-morpholinosydnonimine), S-nitrosothiols (eg. S-nitroso-L-cysteine, S-nitrosoglutathione, S-nitroso-N-acetyl-L-cysteine, S-nitroso-N-acetyl-DL-penicillamine) and 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO). Such donors may be added to a final concentration of between for example 10nM to 300μM. The half-life of the above mentioned donors vary. The half-life of DEA/NO is, for example, approximately 2 minutes. Donors with shorter half-lives, for example 1 to 5 minutes are preferred and those with half-lives of 2 to3 minutes are most preferred.

Determining whether sGC is stimulated may be carried using any suitable method. Typically sGC activity is determined before and after contacting an axon with a substance capable of stimulating sGC. The activity of sGC can be determined directly. It is generally most convenient to do this by measuring the production of cGMP by sGC. For example, by measuring the conversion of radiolabelled GTP into cGMP. Alternatively or additionally, a pH sensitive probe may be used to determine sGC activity, as H^+ ions are also produced by the enzymatic reaction catalysed by sGC. A further method for measuring the activity of sGC is to use a fluorescent tag on the sGC enzyme. In such a method sGC is modified using recombinant DNA techniques so that the sGC comprises a fluorescent polypeptide domain. The fluorescent properties of the resulting sGC: fluoresent polypeptide enzyme change depending on the activity of the enzyme.

It is most convenient to determine whether sGC is stimulated by measuring cGMP levels before and after contacting an axon with a substance that is capable of stimulating sGC. The production of cGMP may be determined by any suitable technique known to those skilled in the field. For example, radioimmunoassays,

enzyme-linked immunoassays (ELISA) and immunohistochemistry may be used. If radioimmunoassays or ELISA are used, typically the total protein content of the tissue is also assayed. In that way the amount of cGMP in a sample can be expressed per amount of protein. Radioimmunoassays, ELISA and immunohistochemistry may all be carried out using anti-cGMP antibodies. Any suitable antibodies may be used. For example, suitable antibodies for use in immunohistochemistry are described in De Vente *et al.* (1987). The above techniques are all well known to those skilled in the art.

cGMP is broken down in cells by the action of phosphodiesterases (PDEs). Therefore, the rate of cGMP accumulation is the difference between its rate of formation by sGC and its rate of destruction by PDEs and if PDE activity is high, cGMP accumulation may not be observed. Thus, PDE inhibitors, for example non-selective PDE inhibitors such as 3- isobutyl-1-methylxanthine (IBMX), may also be added to the assay. In the presence of such inhibitors the rate of cGMP accumulation is equal to the rate of cGMP formation.

The activity of sGC may also be determined indirectly by measuring, for example, the activity of a target of cGMP. Thus, for a viable axon sGC stimulation may be determined by measuring any modulation in the activity of a cGMP target. A number of cGMP targets are known. For example, cGMP activates cGMP dependent protein kinase as well as ion channels. Additionally, the activities of phosphodiesterases are modulated in response to cGMP. Measurement of any of these targets may be used to, indirectly, determine whether sGC is stimulated.

Appropriate control experiments may be carried out when performing the assay of the invention. For example, the assay will be carried out in both the absence and presence of a substance capable of stimulating sGC. Additionally, if cGMP increase or modulation of a cGMP target are measured, the involvement of sGC stimulation may be confirmed by carrying out the assay in the presence of an inhibitor of sGC, for example 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. If sGC is involved in the elevation of cGMP levels in response to NO stimulation, the presence of an sGC inhibitor will reduce the cGMP response observed in the absence of that inhibitor.

A non-viable axon may be assayed to determine whether any sGC stimulation occurs in that axon. An axon may be rendered non-viable by subjecting it to for example, oxygen deprivation and/or sugar, eg. glucose, deprivation. Typically, it is preferable to use conditions under which irreversible damage to the axon occurs. For example, incubating nerves in a medium with no glucose and gassed with 5% CO_2 in N_2 for 1 hour causes irreversible damage to the majority of axons so incubated (Garthwaite *et al.*, 1999).

Other types of cell known to exhibit sGC stimulation and increase in cGMP formation in response to NO may be used as positive controls. For example vascular enodothelial cells show an increase in cGMP formation on stimulation with NO and could therefore be used as positive control in the assay.

Generally, a viable axon is one which shows greater sGC stimulation than that shown by a non-viable axon. Typically, a viable axon will show an increase in sGC activity of at least 2-fold that shown by a non-viable axon. More preferably, a viable axon will show an increase in sGC activity of at least 25-fold, more preferably 50-fold that shown by a non-viable axon.

Similarly, if modulation of activity of a cGMP target is used to measure sGC stimulation, a viable axon is one which shows greater modulation of activity of a cGMP target than that shown by a non-viable axon.

If cGMP generation is used as a measure of sGC stimulation, a viable axon is generally one which shows a greater increase in cGMP generation than that shown by a non-viable axon. Typically, a viable axon will show an increase in cGMP generation of at least 2-fold that shown by a non-viable axon. More preferably, a viable axon will show an increase in cGMP generation of at least 25-fold, more preferably 50-fold that shown by a non-viable axon.

The magnitude of the sGC stimulation observed may depend on the concentration of sGC stimulator present in the assay. Therefore greater sGC stimulation may be observed when higher concentrations of sGC stimulator are used. A viable axon will preferably show sGC stimulation at low concentrations of sGC stimulator.

The invention also provides a method of identifying a substance capable of

protecting an axon from loss of viability, a "protectant". Thus, substances may be identified which preserve axon viability under conditions that would typically lead to axon damage or axon death. Substances identified by such methods may be useful in the prevention and/or treatment of conditions in which damage to or death of axons, in particular CNS white matter axons, is implicated.

Any suitable format may be used for identifying a substance capable of protecting an axon from loss of viability. The assay is, however, typically carried out in an aqueous medium and preferably in a single well of a plastics microtitre plate, so that high through-put screening for protectants may be carried out.

Typically an axon is contacted with a test substance under conditions that, in the absence of the test substance, would lead to a reduction in viability of that axon. Suitable conditions are described above. The viability of an axon may be determined using the viability assay of the invention and this will allow the ability of a test substance to prevent loss of viability to be ascertained.

Suitable control experiments may be carried out. For example, the method may be carried out in the absence of a test substance in order to determine any basal level of sGC stimulation for non-viable axons. Positive control assays may be carried out using the known neuroprotectants, lamotrigine, BW619C89 and BW1003C78 (Xie *et al.*, 1995; Xie and Garthwaite, 1996; Meldrum *et al.*, 1992)

Combinatorial libraries, defined chemical entities, peptide and peptide mimetics, oligonucleotides and natural product libraries may be screened for activity as protectants in assays such as those described above. The candidate substances may be chemical compounds. The candidate substances may be used in an initial screen of, for example, ten substances per reaction, and the substance of these batches which show inhibition tested individually. Suitable candidate substances include antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimaeric antibodies and CDR-grafted antibodies).

A substance which is capable of protecting an axon from a loss of viability, a "protectant", is one which causes a measurable increase in axon viability in the method described above. Preferred substances are those cause an increase in axon viability of at least 10%, at least 25%, at least 50%, at least 100% at least 200%, at

least 500%, at least 1000%, at least 50000%, at least 100000% at a concentration of the protectant of 1μg ml^{-1}, 10μg ml^{-1}, 100μg ml^{-1}, 500μg ml^{-1}, 1mg ml^{-1}, 10mg ml^{-1}, 100mg ml^{-1}. The percentage increase represents the percentage increase in axon viability in a comparison of assays in the presence and absence of the test substance. Any combination of the above mentioned degrees of percentage increase in axon viability and concentration of protectant may be used to define a protectant of the invention, with greater increase in axon viability at lower concentrations of protectant being preferred.

Candidate protectants which show activity in assays such as those described above can then be tested in *ex vivo* models and *in vivo* models. A suitable ex vivo model involves dosing an animal with a neuroprotective agent. After a suitable time for absorption and brain penetration of the agent, the animal is killed. The decapitated head is left at normal body temperature for a given interval (eg. 1h) and then the optic nerves are taken out, incubated in vitro and assayed for viability.

Suitable *in vivo* models include traumatic damage to the spinal cord (which damages white matter). Animal models exist for the majority of the indications given below and are well known to those skilled in the art.

Protectants identified by the screening procedures described above may be used to treat any condition associated with white matter damage. Conditions associated with white matter damage include cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, viral infections (eg. human immunodeficiency virus), alcohol abuse, cerebral malaria and motoneurone disease. Additionally, protectants of the invention may be used in the manufacture of a medicament for use in the treatment of one of the above mentioned indications. The condition of a patient suffering from any of the above mentioned conditions can therefore be improved by administration of such a protectant of the invention. A therapeutically effective amount of a protectant of the invention may be given to a human patient in need thereof.

Protectants of the inventon may be administered in a variety of dosage forms. Thus, they can be administered orally, for example as tablets, troches, lozenges,

aqueous or oily suspensions, dispersible powders or granules. The protectants may also be administered parenterally, either subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques. The protectants may also be administered as suppositories. A physician will be able to determine the required route of administration for each particular patient.

The formulation of a protectant for use in the treatment of a condition associated with white matter damage will depend upon factors such as the nature of the exact protectant, whether a pharmaceutical or veterinary use is intended, etc. A protectant may be formulated for simultaneous, separate or sequential use.

A protectant is typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For example, solid oral forms may contain, together with the active compound, diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, gum arabic, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tabletting, sugar-coating, or film-coating processes.

Liquid dispersions for oral administration may be syrups, emulsions or suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions and emulsions may contain as carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may contain, together with the active compound, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired,

a suitable amount of lidocaine hydrochloride.

Solutions for intravenous administration or infusion may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

A therapeutically effective amount of a protectant is administered to a patient. The dose of a protectant may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular patient. A typical daily dose is from about 0.1 to 50 mg per kg of body weight, according to the activity of the specific protectant, the age, weight and conditions of the subject to be treated, the type and severity of the degeneration and the frequency and route of administration. Preferably, daily dosage levels are from 5 mg to 2 g.

The following Example illustrates the invention.

Example

Materials and methods

Optic nerve preparation

Nerves (about 9 mm long) were excised from adult Wistar rats (240-280 g) after decapitation. They were incubated in Erlenmeyer flasks (50 ml capacity) containing 20 ml of an artificial CSF (aCSF) solution composed of (mM): NaCl (120) KCl (2.0), $CaCl_2$ (2.0), $NaHCO_3$ (26), KH_2PO_4 (1.18), $MgSO_4$ (1.19) and glucose (11), continuously gassed with 95% O_2/5% CO_2. The flasks were held in a shaking water bath at 37°C. For the Ca^{2+}-free medium, ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (1 mM) was substituted for $CaCl_2$ and for the Na^+-free medium, 120 mM choline chloride and 26 mM choline bicarbonate replaced NaCl and $NaHCO_3$ respectively.

Oxygen and glucose deprivation.

After 1-2 h preincubation in aCSF, test nerves were transferred into aCSF lacking glucose and gassed with 5% CO_2 in N_2 for 1 h, a period shown previously to result in irreversible damage to the majority of axons (Garthwaite *et al.*, 1999). Afterwards, the nerves were given a 90 min recovery period in normal aCSF. Modified aCSF and putative axonoprotective compounds were present from 15 min before until 15 min after OGD.

cGMP accumulation

Nerves, with or without a preceding 1 h exposure to OGD (plus 90 min recovery) were exposed to the nitric oxide (NO) donor, DEA/NO (2,2-diethyl-1-nitroso-oxyhydrazine) for 5 min. They were then inactivated in boiling hypotonic buffer and their protein and cGMP contents measured using the automated Lowry method and radioimmunoassay, respectively, as described (Garthwaite & Garthwaite, 1987). The general phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 1 mM) was added 10 min before the exposure to the NO donor, except where indicated. Results are given as means ± SEM and were evaluated using the unpaired Student's *t*-test (2-tailed), $P < 0.05$ being considered significant.

Histology and cGMP Immunohistochemistry

Conventional histology was carried out on semithin sections of resin-embedded nerves as described previously (Garthwaite *et al.*, 1999). For cGMP immunohistochemistry, nerves, with or without various treatments (as described in the text) were fixed in ice-cold, freshly-depolymerised paraformaldehyde (4%) in 0.1 M phosphate buffer (pH 7.4) for 2 h, processed as described before (Southam & Garthwaite, 1993), and then frozen on a cryostat chuck and sectioned at 10 µm intervals. Some nerves were embedded in resin (Durcupan) using conventional methods and cut into 1 µm thick sections. cGMP immunostaining was conducted using a sheep anti-cGMP antibody (Tanaka *et al.*, 1997). Briefly, the sections were incubated with primary antibody (1:80,000) overnight at 4°C . They were then incubated at room temperature with rabbit biotinylated anti-sheep antibody (1:1000; 1 h) followed by Vector stain ABC elite kit (30 min) and then 3.3'-diaminobenzidine

(4 min). Counterstaining was carried out using Mayer's haemalum for 15 s. The 1 μm thick resin-embedded sections were etched with 1:1 mixture of ethanol and saturated sodium hydroxide in ethanol for 5 min before immunohistochemistry; these sections were counterstained with Mayer's haemalum for 5 min.

Materials

The sheep anti-cGMP antibody was a kind gift from Dr. J. de Vente (Maastricht, Netherlands). Secondary antibodies and the ABC kit were purchased from Vector laboratories (Orton Southgate, Peterborough, UK). DEA/NO was from Alexis Corporation (Bingham, Nottingham, UK) or RBI (through Semat Technical UK Ltd., St. Albans, Herts, UK). Tetrodotoxin was from Latoxan Laboratories (Rosans, France). Lamotrigine, BW619C89 and BW1003C87 were supplied by the Wellcome Research Laboratories (Beckenham, Kent). Other chemicals were from Sigma-Aldrich (Poole, Dorset, UK), BDH/Merck (Poole, Dorset, UK) or Tocris-Cookson (Bristol, UK).

Results

Basal cGMP levels in the rat optic nerves averaged 1.06 ± 0.14 pmol/mg protein (n = 4) and the levels were 3-fold higher in presence of the non-selective phosphodiesterase inhibitor, IBMX (1 mM; 3.55 ± 0.36 pmol/mg protein; n = 8). To test the ability of NO to elevate cGMP levels in this tissue, the NO-donor DEA/NO, which dissociates with a half-life of about 2 min (Morley & Keefer, 1993) was used. Exposure of the nerves for 5 min to DEA/NO (10 nM - 300 μM), in the presence of IBMX, resulted in concentration-dependent accumulation of cGMP to levels that were ultimately more than 50-fold higher than in the unstimulated tissue (Fig. 1a). Half-maximal effects occurred at about 10 μM DEA/NO. The inhibitor of NO-stimulated soluble guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (Garthwaite *et al.*, 1995), at a concentration of 3 μM (10 min preincubation), reduced the cGMP response to 100 μM DEA/NO from 219 ± 23 to 32 ± 1 pmol/mg protein (n = 4) confirming the involvement of this enzyme. In the absence of IBMX, maximal cGMP accumulation (with 100 μM DEA/NO), instead of being more than 200

pmol/mg protein, was only 32 ± 3 pmol/mg protein (n = 4), implying a high endogenous phosphodiesterase activity.

Conventional histology of resin-embedded nerves showed that, under control conditions, axons and glial cells (astrocytes and oligodendrocytes) were well preserved in incubated optic nerves (Fig. 2a,d), in agreement with previous findings (Waxman *et al.*, 1992; Garthwaite *et al.*, 1999). To locate the sites of cGMP accumulation, immunohistochemistry was used. In frozen sections from unstimulated nerves (incubated with IBMX), no immunostaining was observed (Fig. 2b). In contrast, exposure to 100 μM DEA/NO (in the presence of IBMX) for 5 min produced powerful staining that was apparently restricted to axons (Fig. 2c). Higher resolution immunohistochemical staining, carried out on semithin sections from resin-embedded nerves, confirmed the staining to be in axons, with no detectable labelling of myelin or glial cells (Fig. 2e,f).

When optic nerves were subjected to 1 h of OGD followed by 90 min recovery in normal aCSF, histology showed abundant axonal swelling (Fig. 2g). The biochemically-measured cGMP response to DEA/NO (100 μM) in nerves previously subjected to OGD was reduced by about 80% (Fig. 1b & 3) and cGMP immunohistochemistry of such nerves showed a marked loss of labelled axons; although there remained a few that stained normally (Fig. 2j).

To further examine the validity of the cGMP response as a marker of axon viability, manoeuvres found previously to reduce or eliminate anoxia-induced loss of the optic nerve compound action potential (Stys, 1998) or OGD-induced axon pathology (Garthwaite *et al.*, 1999) were tested. Complete preservation of the cGMP response was achieved if OGD was imposed in Ca^{2+}-free aCSF or in the presence of the voltage-dependent Na^+ channel inhibitor, tetrodotoxin (TTX, 1 μM); Na^+-free aCSF was less effective, affording only 60% protection (Fig. 1b). Control experiments showed that the cGMP response of nerves exposed to Ca^{2+}-free or Na^+-free aCSF, or TTX, for the same intervals (but without OGD) were normal (n = 4, results not shown). When examined under the microscope, TTX prevented OGD-induced axonopathy (Fig. 2i) and, in parallel, OGD-induced loss of cGMP immunostaining of the axons following exposure to DEA/NO (Fig. 2l). Similar

results were found with Ca^{2+}-free solution (results not shown).

Anoxic damage to optic nerve, assayed using electrophysiology, has been shown to be lessened in the presence of certain antiepileptic drugs (e.g. phenytoin and carbamazepine), local anaesthetics and antiarrhythmic agents (Stys, 1998). The efficacy of these measures is explained by their capacity to block voltage-dependent Na^+ channels. The newer antiepileptic drug, lamotrigine, and the structurally related molecule, BW619C89, block Na^+ channels in a use- and voltage-dependent manner (Xie *et al.*, 1995; Xie & Garthwaite, 1996) and are neuroprotective towards grey matter *in vivo* (Taylor & Meldrum, 1995; Urenjak & Obrenovitch, 1996). Hence, these compounds, and the structurally-related neuroprotectant, BW1003C87 (Meldrum *et al.*, 1992), were tested for their ability to protect the optic nerve against OGD using histology and the NO-stimulated cGMP accumulation.

The compound BW619C89 provided concentration-dependent protection against OGD-induced loss of the cGMP response (Fig. 3), the half-maximal effect being observed at about 6 μM. At the highest concentrations (30-100 μM), the response amplitude was not significantly different from that of control nerves that had not been subjected to OGD. Substantial, though incomplete, protection was also achieved with BW1003C87 (30 μM; 60% protection) and lamotrigine (100 μM; 40% protection) (Fig. 3). On their own, none of the 3 compounds had an adverse effect on the ability of nerves to produce cGMP in response to DEA/NO (Fig. 3 and results not shown). Histology and cGMP immunohistochemistry correlated well with the biochemical results: for example, BW619C89 (30 μM) protected the axons from OGD-induced pathology (Fig. 2h) and loss of axonal cGMP immunostaining (Fig. 2k).

Discussion

The existence of the NO receptor, soluble guanylyl cyclase, in optic nerve was not previously known. Signalling by NO through this mechanism has, however, been described in many other tissues and it appears to be the principal pathway through which physiological NO signalling occurs (Ignarro, 1991; Garthwaite & Boulton, 1995; Christopherson & Bredt, 1997; Hobbs, 1997). The finding that NO

led to cGMP formation specifically in optic nerve axons is surprising for two reasons. First, previous evidence had indicated that, in the CNS, the NO-cGMP signalling pathway is primarily associated with synapses, particularly those mediating glutamatergic neurotransmission (Garthwaite & Boulton, 1995; Christopherson & Bredt, 1997), yet synapses are absent in the optic nerve. Second, the neurones giving rise to the optic nerve axons, the retinal ganglion cells, do not appear to react to NO in the same way because, in bovine or rat retinae, little or no cGMP immunostaining was observed in these cells in response to NO-donor compounds (Gotzes *et al.*, 1998). This may indicate that NO-sensitive guanylyl cyclase is preferentially targetted to the axons rather than to the somatodendritic regions of these particular neurones. Judging by the large enhancement of NO-induced cGMP accumulation brought about by IBMX, the axons are also likely to be rich in phosphodiesterase activity, supporting the possibility that the expression of the guanylyl cyclase there has functional relevance.

Concerning possible sources of endogenous NO in the optic nerve, there is histochemical evidence that guinea-pig optic nerve astrocytes contain an NO synthase enzyme (Qi & Guy, 1996) but we have been unable to detect the endothelial, neuronal or the inducible NO synthase isoforms in glia or axons of the normal rat optic nerve by immunohistochemistry. Staining for the endothelial isoform in endothelial cells themselves, however, was clearly observed (unpublished observations). Thus, NO derived from endothelial cells might constitute the normal effector for the stimulation of cGMP accumulation in optic nerve axons. If so, this would constitute an unusual pathway for intercellular signalling by NO. Additional sources of NO may be present in pathological conditions since, in human glaucomatous patients, the three different NO synthase isoforms are apparently expressed in optic nerve glia (Neufeld *et al.*, 1997), raising the possibility that this pathway is relevant to disorders of optic nerve function in humans. Understanding the functional consequences of cGMP formation in the axons awaits investigation but, in pilot experiments, we have observed that NO-donors elicit a depolarising response from the optic nerve, suggesting a possible action on axonal ion channels (unpublished observation).

cGMP is synthesised from GTP which exists in equilibrium with adenosine 5'-triphosphate (ATP) intracellularly (Voet & Voet. 1995); consequently, non-viable tissue, lacking high energy phosphates, is unable to generate cGMP in this manner, even if the synthetic enzyme should remain intact. The dependence of the cGMP response on cellular viability has been exploited previously for the identification of the sources and targets of NO in the cerebellum (Garthwaite & Garthwaite, 1987). The significant features of the response in the optic nerve were first, its apparently exclusive location in axons and secondly its magnitude, the two together making NO-induced cGMP accumulation a sensitive marker for optic nerve axon viability. Accordingly, in optic nerves previously subjected to 1 h of OGD, the cGMP response was only 17% of its value in control nerves. The residual cGMP elevation was attributable (on the basis of immunohistochemistry) to the survival and normal behaviour of a subpopulation of axons (seemingly distributed randomly), as opposed to a generalised reduction in the ability of axons to generate cGMP. The extent of functional axonal loss recorded with this technique is in excellent agreement with that recorded electrophysiologically, in which 1 h of OGD caused an 80% loss of the optic nerve compound action potential (Fern *et al.*, 1998). Moreover, the various procedures that were found previously to protect optic nerve axons from OGD to differing extents, as judged by a morphometric method (Garthwaite *et al.*, 1999), all had quantitatively very similar effects on the level of NO-induced cGMP accumulation. The correspondence in the readout of two independent methods (one based on histology, the other on function) lends strong support to their reliability for assessing optic nerve axon pathology.

Interpretation of the findings with respect to the mechanism of OGD-induced damage follows that proposed from very similar findings made previously using the quantitative morphometric method (Garthwaite *et al.*, 1999). In brief, the findings indicate that the damage is dependent on the activity of voltage-dependent Na^+ channels and an influx of Ca^{2+} into the axoplasm and are consistent with a mechanism proposed to account for anoxia-induced damage, namely influx of Na^+ followed by reversal of the Na^+-Ca^{2+}-exchanger leading to a Ca^{2+} overload of the axoplasm (Stys, 1998). The lesser protective efficacy of Na^+-free aCSF may be

explained by this manoeuvre itself causing influx of Ca^{2+} which could sum with Ca^{2+} coming in via routes other than the Na^+-Ca^{2+}-exchanger during OGD (Stys & Lopachin, 1998).

Two of the pharmacological agents tested, lamotrigine and BW619C89, have been shown by detailed electrophysiological analysis to be use- and voltage-dependent blockers of voltage-dependent Na^+ channels in central neurones and in cell lines expressing type II Na^+ channels (Xie *et al.*, 1995; Xie & Garthwaite, 1996). The third compound, BW1003C87, is likely to have a similar action since it has a closely related structure and it inhibits glutamate release from brain tissue exposed to the Na^+-channel opener, veratrine, but not the release induced by raised K^+ (Meldrum *et al.*, 1992). All three compounds protect grey matter from ischaemia *in vivo* (Taylor & Meldrum, 1995; Urenjak & Obrenovitch, 1996). In the present study, BW619C89 protected the axons with a potency and efficacy very similar to those registered by morphometric assay (Garthwaite *et al.*, 1999); the degree of protection achieved by the other compounds, at concentrations shown to be maximally effective, also matched those reported by morphometric assay (Garthwaite *et al.*, 1999). The explanation for the differential protective efficacies of the three structurally-similar molecules towards optic nerve axons (BW619C89>BW1003C87>lamotrigine) awaits investigation but it may relate to a differential blockade of the non-inactivating axonal Na^+ channels that appear responsible for much of the Na^+ influx, at least under conditions of anoxia (Stys *et al.*, 1993). Molecules like BW619C89 which appear able to afford a high degree of protection towards both white matter axons and grey matter subjected to ischaemia-like insults, should, in principle, offer superior treatment for conditions such as stroke than strategies (e.g. glutamate receptor blockade) only capable of protecting grey matter.

In conclusion, in the rat optic nerve, the axons selectively and richly express functional NO receptor protein, enabling them to generate large amounts of cGMP in response to NO. While the functional implications of this response remain to be defined, its existence provides a novel, simple and reliable method for quantitatively assessing axonal viability that is likely prove valuable in studies of the pathogenesis of axonal damage and for assessing axonoprotective measures.

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CLAIMS

1. A method for determining the viability of an axon comprising:

(i) contacting the axon with a substance that is capable of stimulating soluble guanylate cyclase (sGC);

(ii) determining whether sGC is stimulated in the axon; and

(iii) determining thereby whether the axon is viable.

2. A method according to claim 1, wherein the axon is a white matter axon.

3. A method according to claim 2, wherein the white matter axon is from the optic nerve, the brain or the spinal cord.

4. A method according to any one of the preceding claims, wherein step (i) is carried out by contacting the axon with nitric oxide (NO), 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), carbon monoxide (CO) or YC-1 and CO.

5. A method according to claim 4 wherein NO is provided in the form of an NO donor.

6. A method according to claim 5, wherein the NO donor is 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO).

7. A method according to any one of the previous claims, wherein step (ii) is carried out by determining whether cGMP generation by the axon increases.

8. A method according to claim 8, wherein the generation of cGMP is determined by radioimmunoassay or immunocytochemistry.

9. A method according to claim 7 or 8, wherein a viable axon is one which shows a greater increase in cGMP generation than that shown by a non-viable axon.

10. A method according to claim 9, wherein the increase in cGMP production is at least 2-fold that shown by a non-viable axon.

11. A method for identifying a substance capable of protecting an axon from loss of viability comprising:

(i) contacting an axon with a test substance under conditions that in the absence of the test substance would lead to a decrease in viability;

(ii) determining the viability of the axon by a method according to any one of the preceding claims; and

(iii) determining thereby whether the test substance can protect the axon from loss of viability.

12. A substance identified by a method according to claim 11.

13. A substance according to claim 12 for use in a method of treatment of the human or animal body by therapy.

14. A substance according to claim 13 for use in a method of treatment of a condition associated with white matter damage.

15. A substance according to claim 14 for use in a method of treatment of cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease.

16. Use of a substance according to claim 11 in the manufacture of a medicament for use in the treatment of a condition associated with white matter damage.

17. Use of a substance according to claim 11 in the manufacture of a medicament for use in the treatment of cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease.

18. A method of treating a host suffering from a condition associated with white matter damage, which method comprises administering to the host a therapeutically effective amount of a substance according to claim 11.

19. A method of treating a host suffering from cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease, which method comprises administering to the host a therapeutically effective amount of a substance according to claim 11.

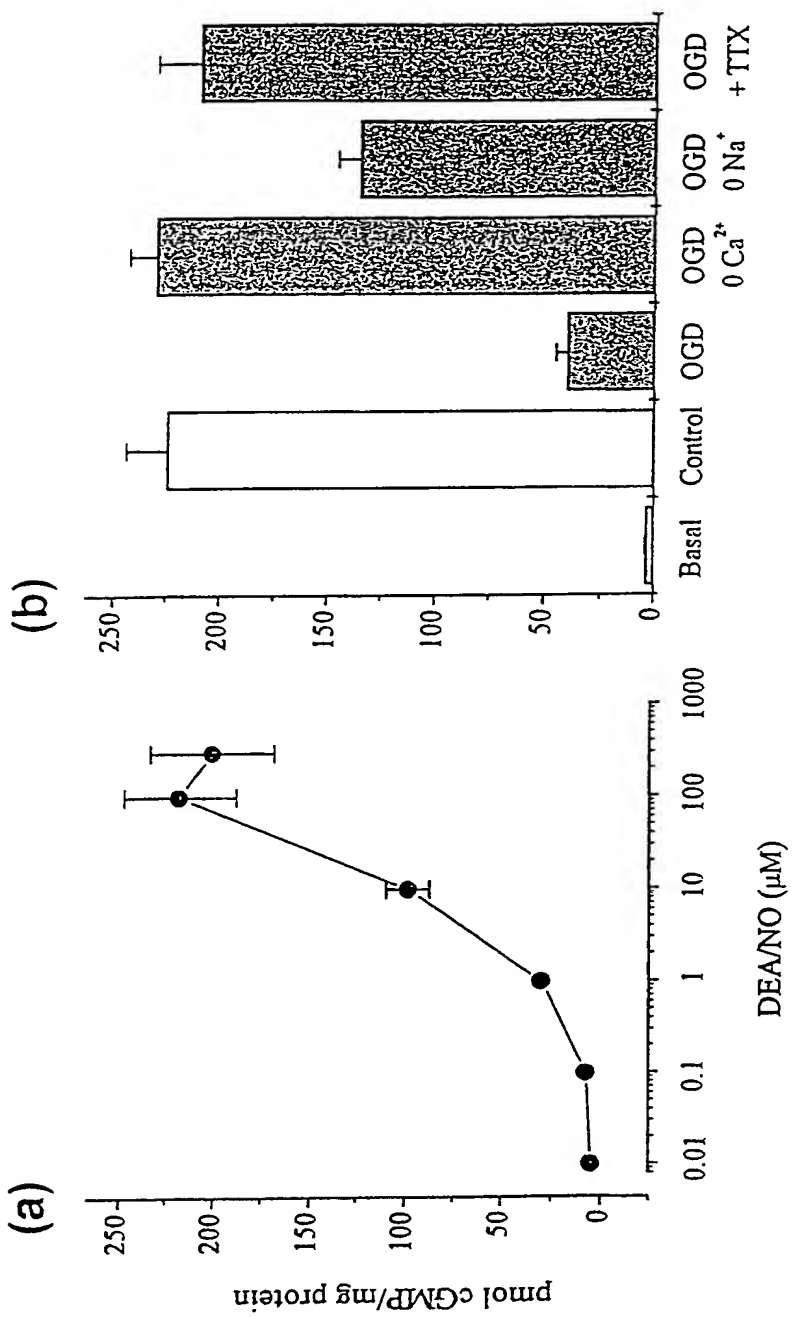
(a)
pmol cGMP/mg protein
250
200
150
100
50
0
0.01
0.1
1
10
100
1000
DEA/NO (μM)
(b)
250
200
150
100
50
0
Basal
Control
OGD
OGD
0 Ca2+
OGD
0 Na+
OGD
+ TTX

Figure 1

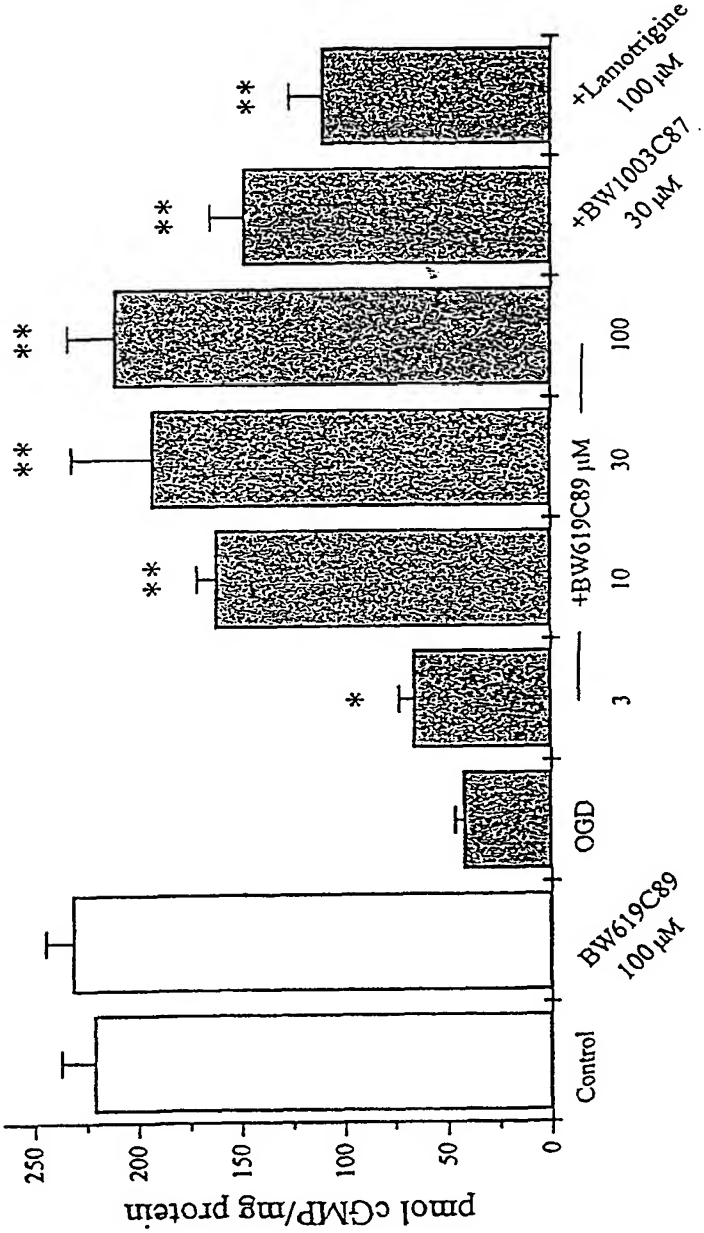
pmol cGMP/mg protein
250
200
150
100
50
0
Control
BW619C89 100 µM
OGD
+BW619C89 µM
3
10
30
100
+BW1003C87 30 µM
+Lamotrigine 100 µM
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Figure 2

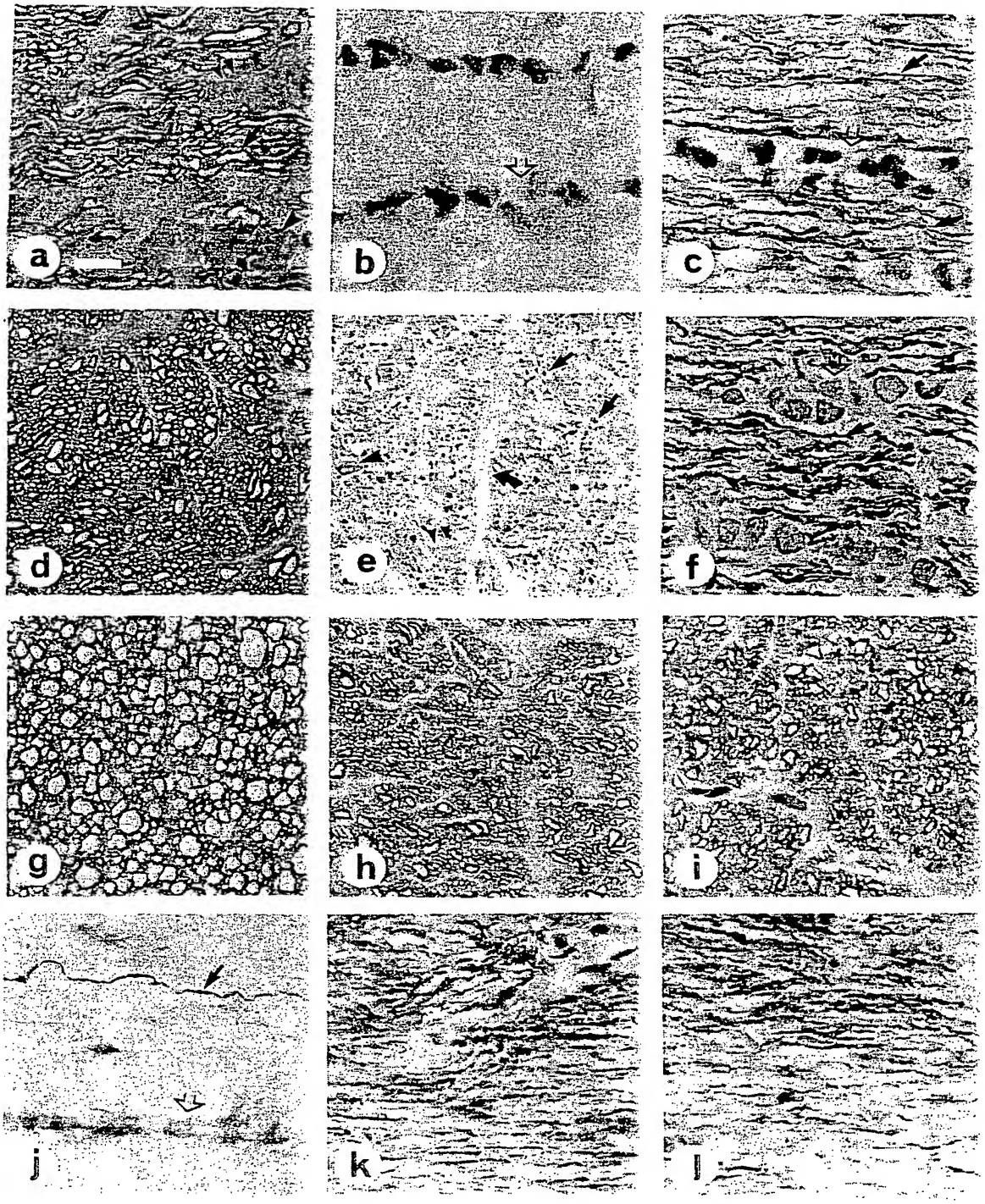

Figure 3